Pharmacological Characterization of Novel Histamine H4 Receptor Ligands

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CHAPTER 05

BIOCHEMICAL AND CELL-BASED FRAGMENT SCREENING IDENTIFIES NOVEL AGONISTS, ANTAGONISTS AND BIASED LIGANDS FOR THE HISTAMINE GPCR FAMILY.

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INTRODUCTION

Fragment-based drug discovery (FBDD) has proven to be an effective starting point to identify hit molecules for efficient drug discovery [240-242]. In contrast to most high-throughput screening efforts using large numbers of relatively complex molecules, the concept of FBDD is the use of a small number of simple chemical structures to better/more efficiently explore the chemical space of the target[240-243]. The fragment library described in the current chapter has been constructed using chemical property filters that are inspired by the "rule of three"[244]: number of heavy atoms ≤ 22, log(P) < 3, number of H-bond donors ≤3, number of H-bond acceptors ≤3, number of rotatable bonds ≤5, number of rings ≥1[245].

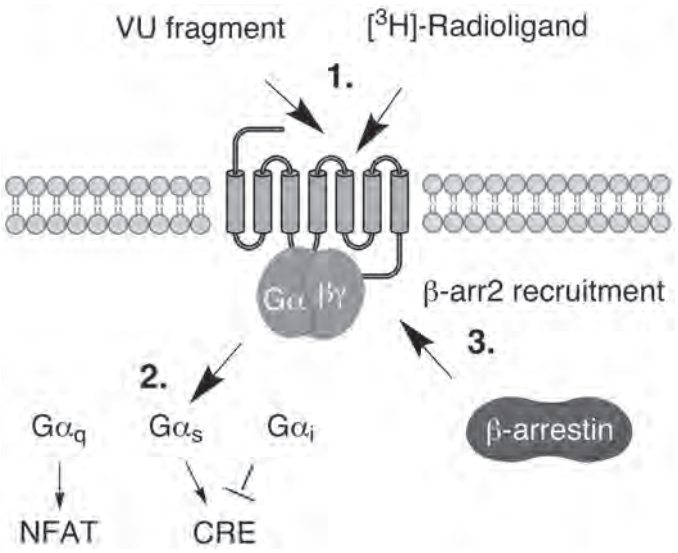
Since fragments are expected to have a relatively low affinity (μM - mM) due to the limited number of possible interactions with the target protein[246], FBDD screening has often been relying on sensitive biophysical techniques[247] (e.g. nuclear magnetic resonance spectroscopy, X-ray crystallography, surface plasma resonance, mass spectrometry)[248] to identify fragment hits. Consequently, the majority of targets in FBDD studies are cytosolic proteins that can easily be purified and interrogated with biophysical methods[249].

At first sight, G protein coupled receptors (GPCRs) seem to be less compatible with FBDD due to their complex integration in the cell membrane, which for long has made them difficult to analyse with biophysical techniques[250,251]. As recently has been reviewed by Keseru *et al.*[252], successful first attempts for FBDD at GPCRs have relied mostly on virtual fragment screening using homology models or GPCR X-ray structures, that have recently become available. Using e.g. the X-ray structure of the histamine H_1 receptor (H_1R), our laboratory recently developed efficient *in silico* protocols to identify new fragment hits with an overall hit rate of 73%[253]. Moreover, with the current expansion in available 3D-X-ray GPCR structures[62,64,67,71,72,74] and consequently the availability of purified GPCR proteins, also this target class is nowadays more amenable, though still challenging, for biophysical FBDD efforts. Experimental FBDD at GPCRs has been pioneered by Heptares Therapeutics using engineered thermostabilized GPCRs (StaRs) (e.g. ADRB1, Adenosine A_{2A}, CXCR4)[254]. Surface plasmon resonance (SPR) screening of A_{2A} and NMR screening of ADRB1 resulted in novel hit identification as well as new lead compounds[255,256].

Despite this progress in FBDD efforts at GPCRs, most biophysical methods are technologically demanding and therefore still not widely applicable. Moreover, purified thermostabilized GPCRs are outside their native lipid environment, potentially representing an artificial receptor entity. Preferably, GPCRs should be in contact with their intracellular signaling partners that are believed to function as allosteric modulators[250,257-259]. We therefore recently set out to test high concentration fragment screening using traditional GPCR radioligand displacement binding assays with GPCR expressing membranes. Using our in-house developed, highly soluble, 1009 fragments-containing library[245], we successfully identified fragment hits on ADRB2, H_1R, H_3R[221], and H_4R[145]. In combination with a chemogenomic approach we were able to identify similarities[260] and differences in fragment binding properties as well as selectivity cliffs[245]. Recently, GPCR fragment screening using fluorescent ligand competition binding assays on adenosine A_1 and A_3 receptors in living cells was shown to be successful as well[250].

It is widely acknowledged that ligand screening at GPCRs based on a single assay readout can miss crucial biochemical information (e.g. agonism/antagonism). Especially the current awareness that GPCRs exhibit so-called biased agonism (e.g. differential G-protein versus β-arrestin coupling in response to chemically distinct agonists) asks for a broader approach in which several assays (covering all downstream effectors) need to be tested to allow for accurate estimation of ligand efficacy[261]. In the present study we therefore focus on two main questions: (I) can we measure *fragment efficacy* on GPCRs in cellular signalling assay? (II) can we detect *biased agonism* at the fragment level?

FIGURE 5-1. Assays used in this study to determine fragment activity profiles.



1) Radioligand displacement assay, 2) Reporter gene assay downstream of G protein signal cascade and 3) β-arrestin2 recruitment (PathHunter assay DiscoveRx).

To investigate fragment efficacy in cellular signalling assays we have made use of the diverse histamine receptor family. The small bioamine histamine (Supplementary figure 5-I), which can actually be considered a fragment itself, binds four GPCRs in the human body (H_1R, H_2R, H_3R and H_4R). The $G\alpha_q$ coupled H_1R [262] and $G\alpha_s$ coupled H_2R [263] have been studied extensively and several marketed antihistamines are available for treating hay fever and peptic ulcer disease, respectively. The newest family members H_3R and H_4R both couple Pertussis toxin (PTx)-sensitive $G\alpha_i$ proteins and bind histamine with a higher affinity than H_1R and H_2R[18,19,83,264]. H_3R and H_4R show considerable amino acid overlap, especially in the transmembrane domains[265]. Interest-

ingly, the H_4R was recently shown to recruit the scaffold protein β-arrestin2 in a PTx-insensitive manner with an enzyme complementation assay[140]. Initially, the well-known H_4R antagonist JNJ 7777120 (Supplementary figure 5-I) was discovered to be a partial β-arrestin biased agonist. Subsequent analysis of a number of known H_4R ligands in this β-arrestin recruitment assay led to reclassification of several H_4R ligands that were either unbiased or showed a preference for G protein signalling or β-arrestin recruitment[140,175,266]. To our knowledge, pharmacological quantification of agonist-induced β-arrestin recruitment by H_1R, H_2R or H_3R has not been published thus far, although the assay is developed and commercially available from DiscoveR$_x$™.
To study the feasibility of biochemical and cell-based assays for FBDD screening on the four histamine receptors, we optimized our standard functional G protein-dependent luciferase reporter gene assays (agonist and antagonist format) to a 384-well homogenous format. PathHunter β-arrestin recruitment assays were performed in parallel and results were compared with fragment screening using radioligand binding assays at each of the four GPCRs.

MATERIALS & METHODS

Materials

[^{3}H]-pyrilamine (25.8 Ci/mmol), [^{3}H]-Tiotidine (82.8 Ci/mmol) [^{3}H]-N$^\alpha$-methylhistamine (85.0 Ci/mmol) and [^{3}H]-histamine (13,4 Ci/mmol) were purchased from Perkin Elmer. All cell culture media for HEK293T cell culture were bought from PAA (Pasching, Austria). Cell culture media for DiscoveRx Pathhunter™ cell lines was obtained from Gibco (Invitrogen).

VU fragment library

The library consists of 1009 fragments that are stored either as powder at 22°C or as 40mM 100% DMSO stock at -20°C. Construction of the library has been described previously[245]. For all assays described a 10 μM final fragment concentration was used. The fragment library is commercially available via IOTA Pharmaceuticals: http://www.iotapharma.com/library.asp

Cell culture and transfection

HEK293T cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 IU/ml penicillin and 50 μg/ml streptomycin at 37°C and 5% CO_2. Cells were seeded $2 \bullet 10^6$ per 10 cm dish one day prior to transfection. Approximately $4 \bullet 10^6$ cells were transfected with 5 μg cDNA using the PEI method.
Transfection for binding assay: 2.5 μg receptor DNA (hH_1R, hH_2R, hH_3R or hH_4R) and 2.5 μg empty pcDEF3 plasmid were mixed with 20 μg 25-kDa linear polyethyleneimine (PEI) in 500 μl 150 mM NaCl.
Transfection for luciferase reporter gene assay: 1 μg receptor DNA (hH_1R, hH_3R or hH_4R) or 2.5 μg (hH_2R) and 2.5 μg NFAT-luciferase (hH_1R) or CRE-luciferase (hH_2R, hH_3R, hH_4R) construct, supplemented with empty pcDEF3 plasmid to a total of 5 μg cDNA, were mixed with 20 μg 25-kDa linear polyethyleneimine (PEI) in 500 μl 150 mM NaCl.

The transfection mixtures were incubated at 22°C for 30 min and subsequently added dropwise to a 10 cm dish with 6 ml new culture medium.
PathHunter cells were cultured in F12 nutrient mixture (HAM) supplemented (H_1R, H_2R and H_3R) or MEM Eagles medium (H_4R) with 10% heat inactivated FBS, 0.2 U/ml penicillin, 0.2 μg/ml streptomycin and 58.4 μg /ml glutamine at 37°C and 5% CO_2. Stable receptor and β-arrestin2 expression was ensured by selection with 800 μg/ml (H_1R, H_2R, H_3R) or 500 μg/ml (H_4R) Geneticin and 300 μg/ml (H_1R, H_2R, H_3R) or 250 μg/ml (H_4R) Hygromycin.

Membrane preparation

Two days post transfection HEK293T cells were washed once with phosphate-buffered saline (PBS) and subsequently scraped from their culture dish in 1 ml of PBS. Cell pellets were collected by centrifugation at ~2000g for 10 min at 4°C and subsequently stored at -20°C until further use.

Radioligand binding assays

The displacement binding assays were performed on homogenized cells expressing H_1R, H_2R, H_3R or H_4R in 50 mM Tris-HCl binding buffer (hH_1R, hH_3R, hH_4R) or 50 mM Sodium/Potassium phosphate buffer (hH_2R) (pH 7.4 at 22°C). These cell homogenates were co-incubated with 10 μM of the fragments and ~K_d concentrations of respective radioligands: [^{3}H]-pyrilamine (hH_1R, K_d= 1.6 nM), [^{3}H]-tiotidine (hH_2R, K_d= 11 nM), [^{3}H]-N$^{\alpha}$-methylhistamine (NAMH) (hH_3R, K_d = 2.9 nM) or [^{3}H]-histamine (hH_4R, K_d = 11 nM) in a total volume of 100 μl/well for 1.5 hrs at 22°C on a shaking table (750 rpm). Bound radioligand was separated from free radioligand via rapid filtration over a 0.5% PEI-pre-soaked glass fiber C plate (GF/C, Perkin Elmer). GF/C plates were subsequently washed three times with ice-cold 50 mM Tris-HCl wash buffer (pH7.4 at 4°C). The retained radioactivity on the GF/C plates was counted by liquid scintillation counting in a Wallac Microbeta (Perkin Elmer). Specific radioligand binding is calculated as the difference between total binding in absence of competitor and aspecific binding in the presence of saturating concentration of competitor (Table 5-1))

Luciferase reporter gene assay

Twenty four hours post transfection HEK293T were seeded in white-bottomed 384-well plates (Greiner) (50.000 cells/well). The next day, cells were stimulated with 10 μl fragments (40 μM) in DMEM supplemented with 1% DMSO. For agonist-mode screening 10 μl DMEM was added per well (for H_3R and H_4R in the presence of 4 μM forskolin), while in the antagonist-mode screening 10 μl 4x concentrated EC_{80} concentration of agonist (Table 1) was added. Cells were incubated for 6 h at 37°C to allow luciferase protein expression. Luciferase activity was measured as previously described[175] although in this new setup we used a homogenous format.

Pathhunter β-arrestin recruitment EFC assay

PathHunter cells co-express H_1R, H_2R, H_3R or H_4R fused to a Prolink/Enzyme Donor and β-Arrestin2 fused to an inactive β-galactosidase (Enzyme Activator (EA)). One day prior to the assay, 20 μl cells were seeded in 384 well, white-walled, clear bottom plates (5.000 cells/well) in recommended plating reagent (DiscoveRx product sheet) and incubated for 24 h at 37°C and 5% CO_2. The assay was started by stimulating the cells with 2.5 μl (100 μM, 3% DMSO) fragments. Depending on the screening mode (agonist or antagonist) either 2.5 μl HBSS +0.1% Bovine Serum Albumine (BSA) or 2.5 μl HBSS+0.1% BSA + 10x concentrated EC_{80} agonist is added (Table 1), respectively. In the antagonist mode, the agonist is added after a 30 minutes pre-incubation at 37°C with fragments. The assay plates were further incubated for 90 minutes at 37°C. Upon recruitment of β-arrestin proteins to the activated GPCR, the Prolink/Enzyme Donor and inactive β-galactosidase come in close proximity and reconstitute into a functional enzyme. Finally, 12 μl detection reagent (i.e. β-galactosidase substrate) was added and the bottom of plates was covered with a white bottom seal. After a 15 min incubation at 22°C, light is measured in a Mithras multilabel reader (1s/well) (Berthold).

TABLE 5-1. Overview radioligands and reference compounds

	Binding		Agonist mode	Antagonist mode	
Receptor	Radioligand	Competitor	Reference agonist	Reference antagonists	Agonist[a]
H_1R	[^{3}H]-pyrilamine (10 nM)	mianserin (10 µM)	histamine (10 µM)	mepyramine (10 µM)	histamine (1 µM / 105 nM)
H_2R	[^{3}H]-tiotidine (2 nM)	ranitidine (10 µM)	histamine (10 µM)	ranitidine (10 µM)	histamine (1 µM / 26 µM)
H_3R	[^{3}H]-NAMH (1 nM)	histamine (10 µM)	histamine / RAMH[b] (10 µM / 10 µM)	thioperamide (10 µM)	histamine / NAMH[b] (316 nM / 200 nM)
H_4R	[^{3}H]-histamine (10 nM)	histamine (10 µM)	histamine (10 µM)	JNJ 7777120 / thioperamide[b] (10 µM / 10 µM)	histamine (100 nM /400 nM)

[a] *EC_{80} values of agonists in reporter gene assay (first value) and PathHunter assay (second value). Agonist concentrations in Pathhunter assay were calculated based on reference data in the product sheets of the cell lines. EC_{80} agonist concentrations in luciferase assay were determined via concentration response curves obtained in 384 well homogenous assay format.* [b] *first compound is used in the G protein assay, second compound is used in the β-arrestin recruitment assay.*

Assessment of assay quality

Z factors[267] for each screening experiment were calculated to control for assay quality and reproducibility (Table 5-2).

TABLE 5-2. Overview Z factors

Receptor	H_1R	H_2R	H_3R	H_4R
Binding	0.76	0.52	0.72	0.68
G protein agonist mode	0.46	0.10	0.50	0.21
G protein antagonist mode	0.65	0.14	0.19	0.58
β-arrestin2 agonist mode	0.34	0.55	0.66	0.41
β-arrestin2 antagonist mode	0.38	0.25	0.37	0.24

[a] *Z factor >0.5 indicates an excellent assay, Z factor between 0-0.5 represents an acceptable assay*[267]

Data normalization and selection of fragment hits

Fragment data was normalized to the amount of specific binding (difference between total binding in absence of reference compound and aspecific binding in presence of reference compound (Table 1)) for each receptor subtype. Cut-off values for hit definition were defined as being more than 2 standard deviations (SD) from fragment average (for H_1R, H_3R and H_4R). H_2R displacement was more scattered (Supplementary Figure 5-II) and therefore fragments that show displacement values that differ more than 4xSD from average are selected as hit. In G protein signalling and β-arrestin recruitment assays, fragment agonism for all receptors was normalized to the maximal response of the endogenous full agonist histamine. Fragment antagonism was percentalized to inhibition of the EC_{80} agonist-induced responses by saturating concentrations of reference antagonists (Table 5-1). Biased fragments are defined by a >25% efficacy in pathway A and a <10% efficacy in pathway B or vice versa.

Definition and assessment of novel hits

All registered entries for H_1R-H_4R ligands were extracted from the ChEMBL database (version 16) and compared with the identified fragment hits. Fragments and H_1R-H_4R ligands were considered as neutrally charged compounds and the most stable tautomer was calculated using Chemaxon's Calculator (version 5.1.4). Tanimoto ECFP-4[268] similarity scores (as calculated by Accelrys' Pipeline Pilot version 6.1.5) <0.4[269] indicated novel fragments. In addition, fragments with new or different functional effects on specific histamine receptor targets than previously reported are also qualified as novel hit.

RESULTS & DISCUSSION

Many aminergic GPCRs endogenously bind small molecules, such as dopamine, adrenaline or histamine, that can be considered fragments. Still, GPCRs have for a long time been largely neglected in the field of FBDD. In this study we set ourselves the challenge to functionally screen a fragment library at the histamine GPCR family. Taken into account the ability of GPCRs to exhibit biased agonism, radioligand binding assays were complemented with two distinct functional responses[261] (i.e. G protein activation and β-arrestin recruitment) (Figure 5-1). This parallel screening strategy allows for the identification of allosteric modulators, agonists, antagonists and biased fragments. In our current study we focus on the applicability of this approach to find multi-target, as well as receptor-specific, functionally active fragments. Furthermore, we aim to use our strategy to identify biased histamine receptor fragments.

Radioligand displacement to determine fragment binding

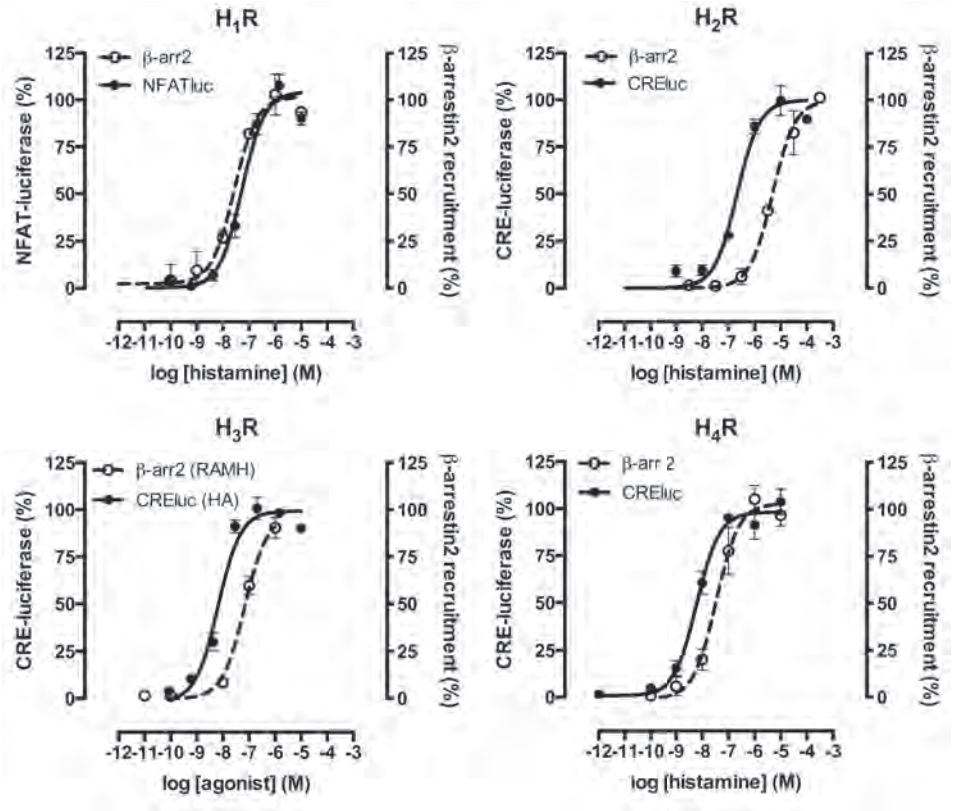
Binding of VU fragments to the 4 different histamine receptors was determined by displacement of high affinity radioligands from HEK293T cell membranes expressing the individual histamine receptor subtypes (Table 5-1). Z factors for the radioligand displacement assays at the four H_1R-H_4R are all > 0.5, which indicates an excellent assay quality[267] (Table 5-2). Percentalized results of the fragment screening are plotted as dot plots in which each dot represents an individual fragment (Figure 5-3A, black dots). Fifty-nine H_1R binders could be identified, 80 fragments bind to the H_2R, 89 bind H_3R and 74 bind H_4R (Table 5-3).

Measurement of fragment antagonism in agonist-induced G protein signalling and β-arrestin recruitment

G protein activation downstream of H_1R-H_4R was measured as NFAT- (H_1R) or CRE- (H_2R, H_3R, H_4R) regulated luciferase activity. β-arrestin2 recruitment was analysed with an enzyme complementation assay (Figure 5-1). Concentration response curves of the endogenous agonist histamine illustrate the successful optimization of the homogenous 384 well assay format for H_1R-H_4R-mediated G protein activation (Figure 5-2). Potency values for histamine (EC_{50} ± SEM values: H_1R 54 ± 6 nM, H_2R 0.2 ± 0.03 μM, H_3R 6.6 ± 1 nM, H_4R, 6.2 ± 1 nM) were comparable to previously reported non-homogenous 96 well formats[123] (data not shown). In addition, concentration response curves of the references agonists (Table 5-1, Figure 5-2) in the β-arrestin2 recruitment assay show comparable potency values as indicated by the manufacturer: EC_{50} values: H_1R 27 ± 7 nM, H_2R 4.8 ± 0.6 μM, H_3R 69 ± 6 nM, H_4R, 34 ± 6 nM.

Fragment antagonism was determined in the presence of EC_{80} concentrations of histamine (Table 5-1). Normalized results (H_3R) are shown in figure 5-3A. Comparing both antagonist assays and binding hits for the histamine H_3R, overlap and differences in hits are visible (Figure 5-3A). Similar trends were observed for H_1R, H_3R and H_4R (data not shown). Also, functionally active fragments that did not displace radioligand binding were found, which could potentially hint at allosteric fragments (i.e. fragments that modulating receptor functioning via a different interaction site than the orthosteric ligand site[44]).

FIGURE 5-2. Assay validation.



Concentration response curves of reference compounds in G protein signalling (●) and β-arrestin recruitment (O) assays at the four histamine receptor subtypes. Representative curves are shown from at least two experiments performed in duplicate. Graphs are fitted two a three variables concentration response curve. Error bars indicate SEM values. HA, histamine; RAMH, (R)-α-methylhistamine.

A large number of fragments inhibited histamine-induced responses in the G protein assays for the four histamine receptor subtypes. In total, 385 fragments antagonized the agonist-induced H_1R signalling, 325 fragments H_2R signalling, 434 fragments H_3R signalling and 217 fragments H_4R signalling. Interestingly, The number of hits was considerably lower for fragments that inhibited agonist-induced β-arrestin recruitment to H_1R (90 fragments), H_2R (27 fragments), H_3R (197 fragments) and H_4R (96 fragments). The assay quality of the antagonist mode screens is reflected by the respective Z factors[267] (Table 2). For the G protein luciferase assay, H_1R and H_4R screens have a Z factor >0.5, which indicates an

excellent assay quality. However, in this assay H_2R and H_3R screens have Z factors <0.2 indicating moderate assay quality. Z factors from the β-arrestin recruitment assays were <0.4, which means that also these assays are of moderate quality. Only fragments with confirmed receptor binding are considered in further hit analyses to eliminate possible false positive hits that result from off-target signalling or assays Z factor <0.5 (see results overview section below).

Measurement of fragment agonism in G protein signalling and β-arrestin recruitment to histamine receptors

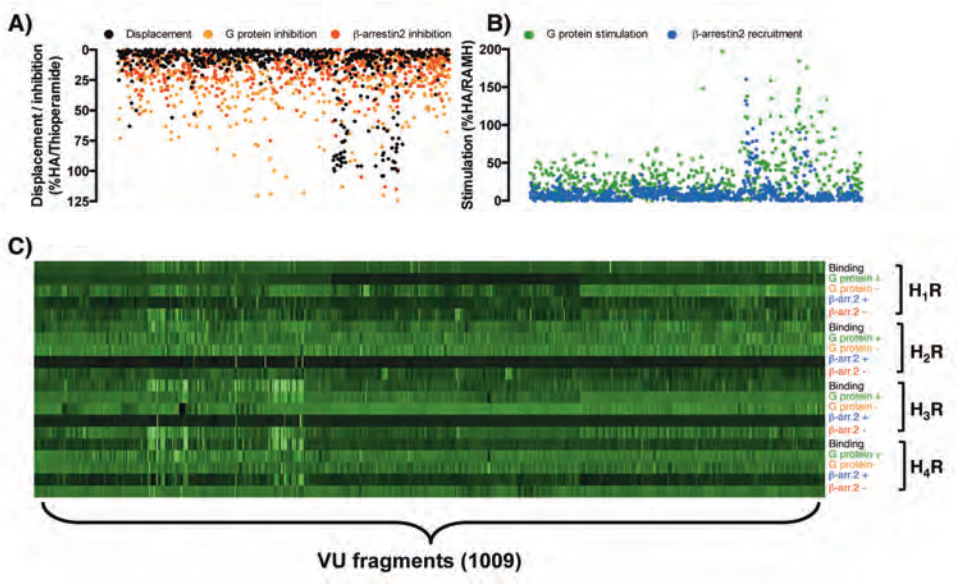
Next, fragment agonism in G protein signalling and β-arrestin recruitment was measured and percentalized to the maximum signal of respective reference full agonist (Table 5-1). In the G protein assay, 3 fragments activated H_1R, 289 stimulated H_2R, and 48 and 240 fragments induced H_3R and H_4R signalling respectively. In the β-arrestin recruitment assay 65 H_1R hits, 5 H_2R, 7 H_3R and 65 H_4R hits were identified. An overlap in fragment agonist hits between G protein signalling and β-arrestin recruitment to the histamine H_3R could be observed, however also clear differences can be seen (Figure 5-3b). Similar trends in overlap and variety were observed for H_1R, H_2R and H_4R. Z factors[267] for the G protein-mediated H_1R-H_4R agonist screening assay varied from moderate (H_2R, H_4R Z factor <0.2) to good (H_1R, H_3R Z factor >0.4). The H_1R-H_4R β-arrestin recruitment agonist screen quality is moderate (H_1R, H_4R, Z factor <0.4) or excellent (H_2R, H_3R, Z factor >0.5) (Table 5-2).

Result overviews

A schematic data overview of five screening assays at the four histamine receptor subtypes is given in a heat map in which the fragment results are coloured based on their percentage displacement or efficacy (Figure 5-3c). Two clear trends can be observed in the heat map: 1) Less fragments induced β-arrestin recruitment (as illustrated by the almost black horizontal lines for β-arr+ at H_2R, H_3R and H_4R) as compared to G protein signalling when screened at 10 μM concentration. This can be explained by the fact that reporter gene assays are at the end of the signalling cascades and known for their signal amplification, whereas GPCR β-arrestin interactions follow a 1:1 stochiometry[175,270]. Moreover, reporter gene transcription can also be the consequence of non-GPCR mediated signalling and hence fragments that target other proteins in this signalling cascade can cause false positives (validation studies are currently on-going to filter out the non-receptor mediated effects). 2) An overlap between H_3R and H_4R binders can be observed at the left site of the heat map (Figure 5-3c). This is possibly due to the reasonably high amino acid similarity in the H_3R and H_4R binding pocket[271].

Considering the large amount of hits in the functional assays (i.e. G protein luciferase assay) in combination with moderate Z factors, we set a clear threshold for further hit analyses. Only fragments that displace radioligand binding from histamine receptors (Z factors>0.5) *and* have efficacy in at least one functional assay are analysed and discussed in the subsequent sections of this chapter.

FIGURE 5-3 Fragment screening results.



A) Overview of [³H]-RAMH displacement binding (●), inhibition of histamine-induced Gα_i protein signalling (●) and inhibition of RAMH-induced β-arrestin2 recruitment (●) to the H3R by 1009 VU fragments. Each dot represent a single fragment B) Overview of fragment-induced G protein activation (●) and β-arrestin2 recruitment (●) to H3R. C) Heat map of all fragment data points for all four histamine receptors. Fragments are positioned horizontally and the five different assays for each receptor subtype are positioned vertically. Highest value is bright green, lowest values is black.

Fragment hits at the histamine receptor family have diverse chemical properties

Identified hits at the four different histamine receptor subtypes are chemical diverse fragments and do not form clusters based on heavy atom count, molecular weight, log(P), complexity[272] and cyclicity[272] (data not shown). This is illustrated in the distribution plots in Figure 5-4, in which for each receptor subtype the heavy atom count is plotted against the complexity of each fragment. The complete library is shown in grey, whereas each fragment that shows binding and activity in one of the assays is coloured depending on its functional activity. Based on the distribution pattern we can conclude that our screening strategy identifies a variety of fragments with different chemical properties. The average complexity value of the hits for each receptor subtype is comparable and ranges from 0.621 to 0.690. The heavy atom count is in the same range for H_1R, H_2R hits (16.8 and 16.6) and H_3R, H_4R hits (15.1 and 15.7). In general, the agonist hits have a lower complexity and heavy atom count than identified antagonists (Figure 5-4).

TABLE 5-3. Overview of fragment hit numbers

GPCR	Total hits	Displacers with efficacy	Antagonists (both assays)	Agonists (both assays)	Possibly G protein biased	Possibly β-arrestin biased	Non-functional displacers	Novel fragments[a]
H_1R	59	55	39	1	1	8	4	26
H_2R	80	54	2	4	18	0	26	13
H_3R	89	76	20	7	26	0	13	30
H_4R	66	63	7	23	3	23	3	18

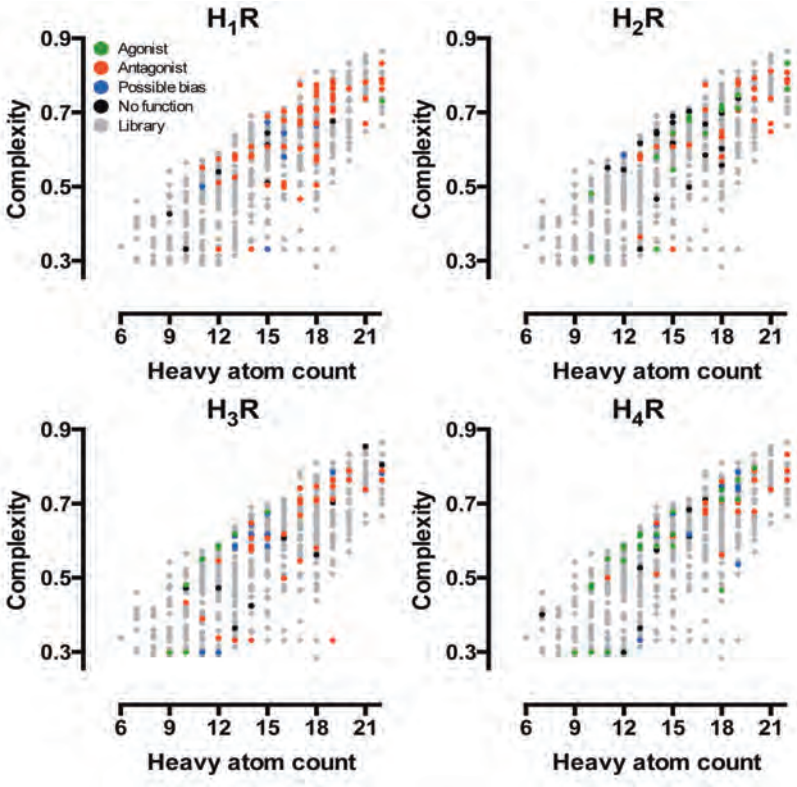
[a] *novel hits are fragments that are to the best of our knowledge are not known to bind to the indicated target or fragments that show not yet reported functionality (changed or biased efficacy).*

Overlap and specificity in fragment hits that bind H_1R-H_4R

Fragment hits identified in the radioligand binding assay are ordered in a Venn diagram to illustrate fragment specificity or fragment overlap between different histamine receptor subtypes. Ninety-three fragments bind to only one histamine receptor subtype (H_1R, 24 hits; H_2R, 40 hits; H_3R, 23 hits and H_4R, 9 hits) (Figure 5-5). This illustrates that our fragment library can distinguish four closely related targets and is very useful if target specificity is required (i.e. to prevent off target binding and associated side effects). Apparently, amino acid differences in the ligand-binding pocket between the H_1R-H_4R allows identification of specific binders. In addition, overlapping fragment hits were found as well (H_1R, 35 hits; H_2R, 40 hits; H_3R, 66 hits and H_4R, 57 hits) that target two or more histamine receptors. In total, 82 fragments showed multi-target binding (Figure 5-5). This is not surprising considering the fact that all four receptor subtypes bind the same fragment (i.e histamine (Supplementary figure 5-I)) as their endogenous ligand. However, also overlap in fragment hits between different protein families has been identified (e.g. H_4R and the serotonin ion channel $5HT_{3A}$[260]). FBDD might therefore also be very suitable as starting point for the development of multi-target drugs[273].

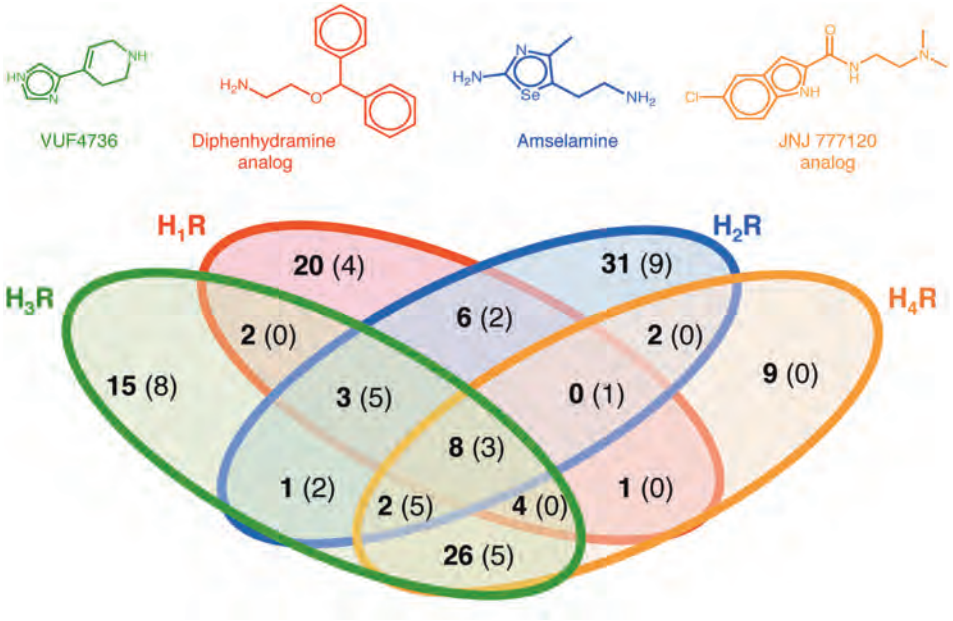
Structures from specific H_1R-H_4R ligands (analogues) were identified amongst the fragments hits, which illustrates the validity of the radioligand displacement screening assay. Analogs of H_1R-specific antagonist diphenhydramine (red) and H_4R-specific indolecarboxamide JNJ 7777120 (Supplementary figure 5-I) (orange) as well as H_2R-specific agonist amselamine[274] (blue) and H_3R ligand VUF4736 were indeed retrieved as hit in the binding screens (Figure 5-5). Only fragments that displaced the radioligand and showed >25% efficacy in agonist or antagonist screens were further considered in this manuscript.

FIGURE 5-4. Diversity of the fragment library and distribution of identified hits.



Fragments are plotted based on their heavy atom count and their complexity[1]*. Each grey dot represents a fragment from the VU fragment library, whereas fragments that displaced the radioligand and exhibited efficacy in at least one functional assay are coloured: agonists (●), antagonists (●), biased fragments (●), no function (●).* [1]*complexity is defined by four structural descriptors: (1) the maximum number of the smallest set of smallest rings (sssrs), (2) the maximum number of heavy atoms, (3) the maximum number of bonds, where covalent bonds between hydrogen atoms and other atoms are excluded and, (4) the maximum sum of heavy atomic numbers*[272]*.*

FIGURE 5-5. Venn diagram of fragments that displace radioligand from histamine receptors.



Numbers of receptor-specific and receptor-overlapping fragments are indicated in the appropriate overlapping area. In bold: fragment hits with functional efficacy; Between parentheses: non functional fragment hits. Indicated chemical structures are known specific ligands for each histamine receptor subtype.

Identification of (novel) histamine receptor antagonists

Sixty-eight fragments antagonized both agonist-induced G protein signalling and β-arrestin recruitment for at least one of the four histamine receptor subtypes (Figure 5-6A). Fragments resembling known H_1R-H_4R antagonists were recovered in our hit selection, such as an analog of H_1R antagonist diphenhydramine[275] (Supplementary figure 5-I) and the H_3R / H_4R dually active antagonist thioperamide[85,86,276] (Supplementary figure 5-I) (data not shown).

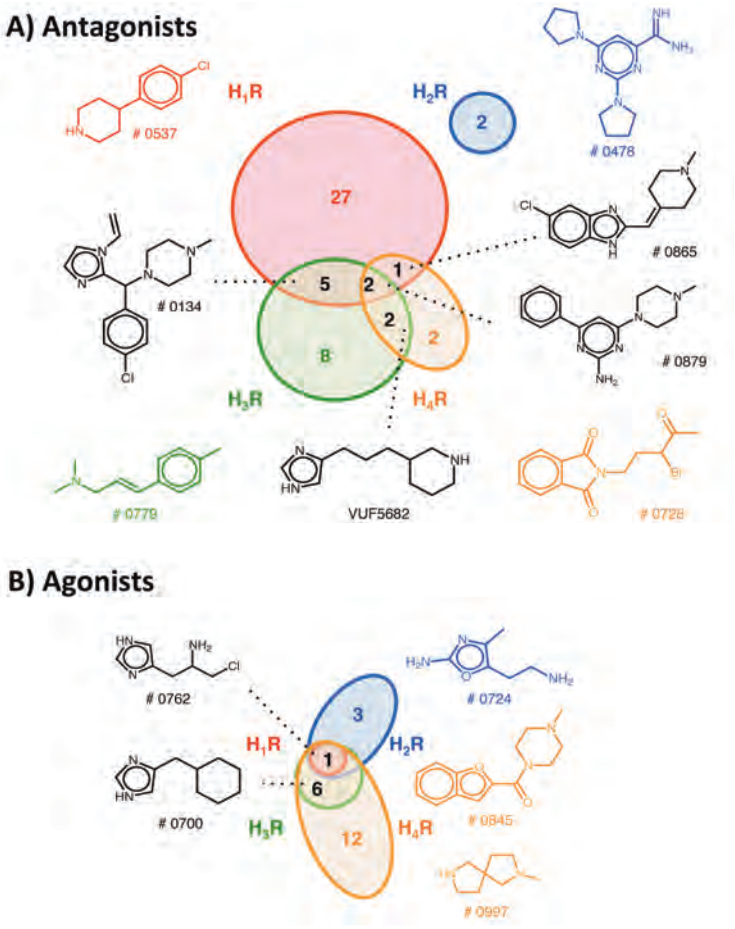
Twenty-seven fragments specifically inhibited histamine-induced H_1R signalling to G_q and the H_1R-mediated recruitment of β-arrestin. Twelve of these fragments resemble known tricyclic H_1R antagonists such as clozapine, diphenhydramine and ketotifen (Supplementary figure 5-I) or have an ECFP-4 score >0.4[269] and are therefore not recognized as new H_1R scaffold. Two fragments are a substructure (Supplementary figure I, #0039) of the typical H_1R antagonist diphenhydramine (Supplementary figure 5-I), which could indicate that only half of the diphenhydramine structure is needed to ensure antagonism. Although four other fragments resemble H_1R antagonists and fit well in the pharmacophore model, the similarity score is

<0.4[269] and consequently these scaffolds are marked as novel. In total, thirteen novel H_1R specific antagonist structures were found (e.g. #0537, figure 5-6a, red).
Two H_2R-specific antagonists were identified that are both novel structures. One fragment is much smaller than known H_2R compounds and does not possess H_2R antagonist-specific features (supplementary figure 5-I, #0674). Fragment #0478 (Figure 5-6a, blue) was previously shown to interact with the β2 adrenergic receptor (ADRB2)[245], ,which is surprising given the relatively low ligand overlap and ligand similarity between these receptors compared to other aminergic GPCRs[271]. The binding site similarity between H_2R and ADRB2 is however as high as the similarity between H_2R and H_1R and significantly higher than the similarity between H_2R and H_3R/H_4R[271]. Moreover, H_2R mutation studies have indicated that the affinity of H_2R for ADRB2 ligands can be increased by small changes in the H_2R binding pocket[263,277].
Eight H_3R-specific antagonists were found that did not contain an imidazole. Three of these fragments represent novel antagonist scaffolds that fit into the H_3R antagonist pharmacophore model (i.e. two positive nitrogen atoms separate by a aromatic moiety[278]). One novel fragment contains only one positive nitrogen (Figure 5-6A, #0779, green)
Two H_4R-specific antagonists were identified of which one was previously published as VUF5681 (supplementary figure 5-I), a H_3R antagonist that preferentially targets H_3R over H_4R in an antagonistic manner[279]. Remarkably, this compound stimulated G protein signalling by the H_3R. The novel H_4R-specific antagonist (Figure 5-6A, #0728, orange) represents a scaffold that does not possess common H_4R pharmacophore features as described by Istyastono et al[155]. In this pharmacophore, an aromatic moiety is on one side directly connected to a H-bond donor and on the other side a H-bond is at 4-6Å distance. Two liphophilic/aromatic moieties are optional side groups[155].

Identification of (novel) multi-target antagonists
A growing trend in medicinal chemistry is the dual function or multiple activities of potential drugs (i.e polypharmacology) [280-283]. Often two pharmacophore structures are linked to ensure dual target activity. However such structures are large and do not always possess desirable drug-like properties. Structure identification of fragments that target dual or multiple receptor subtypes can serve as starting points for the development of such multi-target compounds[284-286].
Dual activity H_1R/H_3R antagonists in G protein signalling and β-arrestin recruitment can be divided in two quinazoline structures, a quinoxaline and two piperazine-containing fragments that both have an aromatic moiety and one also has an imidazole moiety (Figure 5-6A, #0134 black). The quinoxaline structure is not known to inhibit agonist-induced signalling of H_1R and therefore represents a novel fragment for this receptor.

One fragment antagonized both H_1R and H_4R (Figure 5-6A, #0865, black). This fragment resembles JNJ 7777120 (Supplementary figure 5-I), a specific H_4R antagonist that was recently discovered to exhibit bias agonism toward β-arrestin recruitment. Interestingly, an extensive study into JNJ 7777120 analogous revealed that most indolecarboxamides are able to induce

FIGURE 5-6. Venn diagrams of unbiased antagonists and agonists.



Numbers of receptor-specific and receptor-overlapping fragments are indicated in the appropriate overlapping areas. Fragments displace the respective radioligand and have efficacy in both G protein activation and β-arrestin2 recruitment. Shown fragment structures are examples of novel fragments with specific or overlapping efficacy. A) Fragment hits from the antagonist screens. B) Fragment hits from the agonist screens.

β-arrestin recruitment to the H_4R. The antagonism on H_1R is a thus far unknown property of this fragment, which could function as a novel lead fragment for dual H_1R/H_4R antagonists. H_1R/H_4R antagonist combination therapies for diseases as pruritus or allergic contact dermatitis have been suggested previously[287,288]. The above mentioned dual activity compounds could be a very interesting approach to target both receptor simultaneously with a single compound.

Two dual active imidazole-containing H_3R/ H_4R antagonists were identified. One of them is the well-known H_3R/H_4R dual activity inverse agonist thioperamide (Supplementary figure 5-I). The other fragment (Figure 5-6A, #0703, black) is VUF5682, for which H_3R antagonism and H_4R binding has been reported[279].

Two fragments act as antagonists on H_1R, H_3R and H_4R. Aminopyrimidine #0879 (Figure 5-6A, #0879, black) has been previously published as selective H_4R antagonist[289]. The other fragment has only been shown to bind H_4R, but no functional data is reported thus far. For both fragments, no efficacy values are published for H_1R and H_3R. The fact that we observe antagonism at all three receptors could indicate that these fragments could be multitarget histamine receptor inhibitors. Recently, it was reported that three histamine receptors (H_1R, H_3R and H_4R) play a role in pruritus. Triple target fragments could therefore be an interesting option for treating itch[290]

Identification of known and novel histamine receptor agonists

Fragments that displace radioligand binding from histamine receptors and are able to activate both G protein signalling and β-arrestin recruitment to a similar extent at 10 μM are plotted in a Venn diagram to visualize overlap and specificity (Figure 5-6b). Known H_1R-H_4R agonists were present in the final hit selection, such as H_2R agonist amselamine[274] and H_3R/H_4R dual active compound immepip[291]. No fragments were found that specifically activate H_1R or H_3R.

Three H_2R specific agonists could be extracted from our screen. Amselamine is a known H_2R-agonist (Figure 5-5), whereas the other hits are closely related to this fragment but have to our knowledge not been reported previously. Replacing the selenium atom from amselamine with an oxygen atom results in a novel H_2R compound (Figure 5-6B, #0724, blue). Extending amthamine (supplementary graph 5-I) with a fenol on the amine did not change the functional profile of the third fragment.

Thirteen fragments specifically target H_4R as agonist. Ten of these fragments also bind to H_3R, but have no effect or a different efficacy on this receptor (see bias agonism). One piperidine containing fragment has been previously shown to bind to H_4R as well as 5HT3a[260], but here is the first time that H_4R agonism is described. Three novel H_4R agonists were identified: one fragment possesses typical histamine receptor elements (i.e. imidazole ring, basic nitrogen), whereas the 2-methyl-2,7-diazaspiro[4,4]nonane ring system of H_4R agonist #0997 (Figure 5-6b, #0997, orange) has previously been tested as alternative for the basic methylpiperazine in quinazoline H_4R ligands[125]. There the substitution resulted in a decreased affinity and was not further investigated. It is now interesting to see that the 2-methyl-2,7-diazaspiro[4,4]nonane fragment itself is able to bind and activate the H_4R. Considering the bioisosteric overlap of #0997 with methylpiperazine, it would be interesting to investigate the functionality of that basic group. The third novel H_4R agonist fragment is a JNJ 7777120 analog (Figure 5-6b, #0845, orange). Previously, we observed that a JNJ7777120 analog with a nitro group on the aromatic indole moiety could activate hH_4R-mediated G protein signaling and β-arrestin recruitment[266]. This is now the second

indolecarboxamide analog that is an agonist in both G protein and β-arrestin signaling downstream of H_4R, which highlights the narrow margin between agonist and antagonist structural features in this chemical class.

Identification of multi-target agonists

Fragments with multitarget agonism can function as useful tool compounds to unravel GPCR activation mechanisms. Fragment #0762, which closely resembles histamine is an unbiased agonist at all four histamine receptor subtypes (Figure 5-6b, #0762, black).

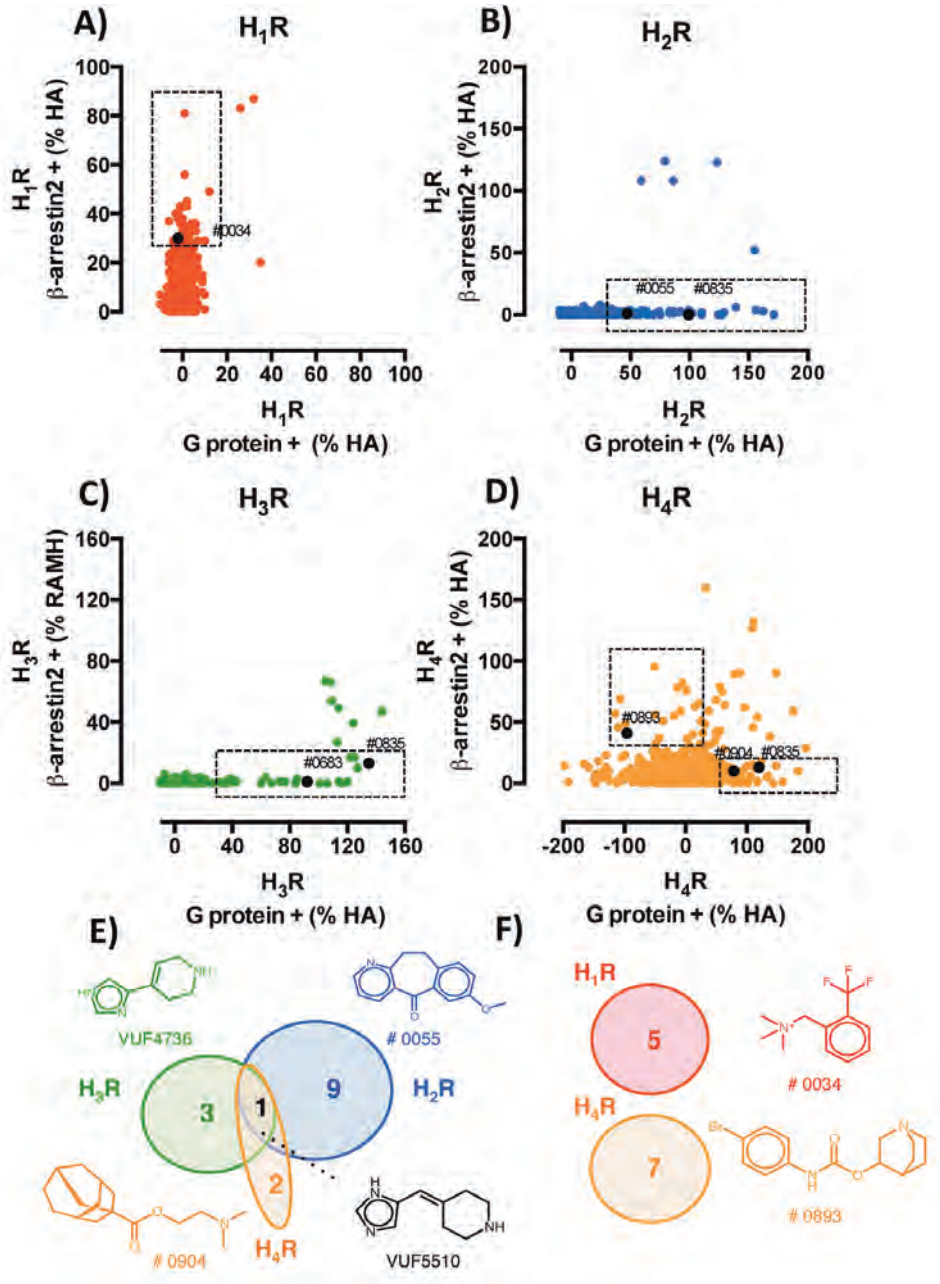
Six fragments showed dual agonism at both H_3R and H_4R. Six hits were previously identified as dual agonist and represent known structures as imbutamine, homohistamine, immepip and immethridine or closely related analogs[130,292] (Supplementary figure 5-I). Two fragments are intriguing H_3R/H_4R agonist hits as they only contain an imidazole and do not contain a basic amine group (or pyridine) at the end of the alkyl chain (Figure 5-6b, #0700, black). This would suggest that the basic amine is not crucial for agonism in these receptor subtypes, an observation that was previously highlighted for the H_3R[293], but has not been published for the H_4R. Fragment #0700 has been reported previously as dual $H_4R/5HT_{3A}$ binder, but here we show for the first time that this fragment can activate both H_3R and H_4R.

Identification of novel histamine receptor- specific biased agonists

The use of two functional readouts for each histamine receptor subtype allows for the identification of G protein or β-arrestin-biased fragments. An efficacy correlation plot for each receptor illustrates the fragment agonist efficacy for both pathways (Figure 5-7A-D). Based on these plots, biased fragments can be identified that show efficacy in one assay but are (nearly) inactive in the other assay (see M&M for details). Remarkably, a different correlation pattern can be observed for each histamine receptor subtype. More fragments stimulated H_1R -mediated β-arrestin recruitment than H_1R -mediated G protein activation (Figure 5-7A). For both H_2R and H_3R this trend is the opposite and more G protein activation than β-arrestin recruitment was observed upon stimulation with fragments. In fact, no β-arrestin-biased fragments were found for H_2R and H_3R receptors (Figure 5-7B, C). In contrast, H_4Rs have both G protein as well as β-arrestin biased fragments. Moreover, a large fragment group showed inhibition of the basal H_4R signalling and could possibly be inverse agonists (Figure 5-7D).

The strict separation between β-arrestin-biased hits (H_1R) on one hand and G protein biased hits (H_2R, H_3R) on the other hand seems to have different causes. First, the absence of histamine H_1R G protein-biased agonists is not surprising considering the low numbers of unbiased H_1R agonists. However, the fact that we observe β-arrestin-biased H_1R hits might indicate that β-arrestin recruitment requires less conformational (intermolecular) rearrangements than G protein coupling and activation. This hypothesis is in line with our earlier work on β-arrestin biased H_4R ligands, in which we suggested that β-arrestin biased ligands seem to induce less conformational changes due to less interaction points than unbiased ligands[266]. Secondly, a possible explanation for the absence of H_2R/H_3R β-arrestin biased agonists could be that

FIGURE 5-7. Efficacy correlation plots and Venn diagrams of biased histamine receptor agonists.



<< Fragments that displace radioligand binding from histamine receptors and show efficacy in one pathway, but are (nearly) inactive in the other are classified as biased agonists. For each histamine receptor biased agonists could be identified (A-D). Venn diagrams show specificity and overlap in biased fragment hits for the receptor subtypes. E) G protein biased fragment hits. F) β-arrestin biased fragment hits. Fragment structures shown represent examples of novel histamine receptor fragments and their positions in the correlation graphs are indicated with black dots.

β-arrestin recruitment for these subtypes is predominantly the consequence of G protein activation, rather than an independent downstream effect. Consequently, no full β-arrestin biased fragments were identified for H_2R/H_3R, although β-arrestin recruitment can be observed and measured for unbiased agonists.

Nine G protein biased fragments were found for the H_2R (Figure 5-7B and E), of which six fragments represent novel H_2R scaffolds (Figure 5-7E, #0055, blue).
Three G protein-biased fragments are H_3R specific (Figure 5-7C and E). Fragment VUF4736 has been previously described as H_3R agonist in a CRE reporter gene assay[279], but we now show that this fragment is not able to induce β-arrestin recruitment to the H_3R (Figure 5-7E, green).
Moreover, also two G protein-biased fragments could be identified for the H_4R (Figure 5-7D and E). Fragment #0904 is an unknown scaffold for the histamine receptor family and could function as an interesting tool compound to study G protein biased H_4R signalling (Figure 5-7E, #0904, orange).
Interestingly, one fragment (VUF5510) exhibited G protein biased agonism on three receptor subtypes (i.e. H_2R, H_3R and H_4R) (Figure 5-7E, black). This fragment was previously shown to activate the H_3R, but no efficacy was yet determined on H_2R and H_4R [279].
Five fragments showed β-arrestin-biased efficacy on the H_1R. In addition, these fragments inhibited the histamine-induced agonism in a G protein assay and can thus be stated as full biased fragments (Figure 5-7A and F). These structures are novel structures for the H_1R. Two of these fragments possess a CF3 substituent on the aromatic ring and resemble 2-(3-trifluoromethylphenyl) histamine (Supplementary figure 5-I), a previously described guinea pig H_1R agonist[294] (Figure 5-7F, #0034, orange).

The H_4R is the only histamine receptor for which ligand bias has hitherto been evaluated and our fragment screening at the H_4R shows the largest group of β-arrestin-biased ligands (Figure 5-7d). Seven fragments specifically target the H_4R in a β-arrestin-biased fashion. In addition these fragments show no functional activity or antagonism in a G protein assay, indicating that the observed fragments are fully biased. Interestingly, only three of these fragments are closely related to the previously discovered β-arrestin-biased agonist JNJ 7777120, whereas the others represent novel structures. Fragment #0893 (Figure 5-7F, #0894, orange) is a yet unknown structure that could be an interesting lead compound to further explore the basis for H_4R biased signalling. For all four histamine receptors, no data is yet available concerning

the physiological effects of biased signalling. Hence, the here-identified compounds can be valuable tools to specifically unravel these downstream pathways.

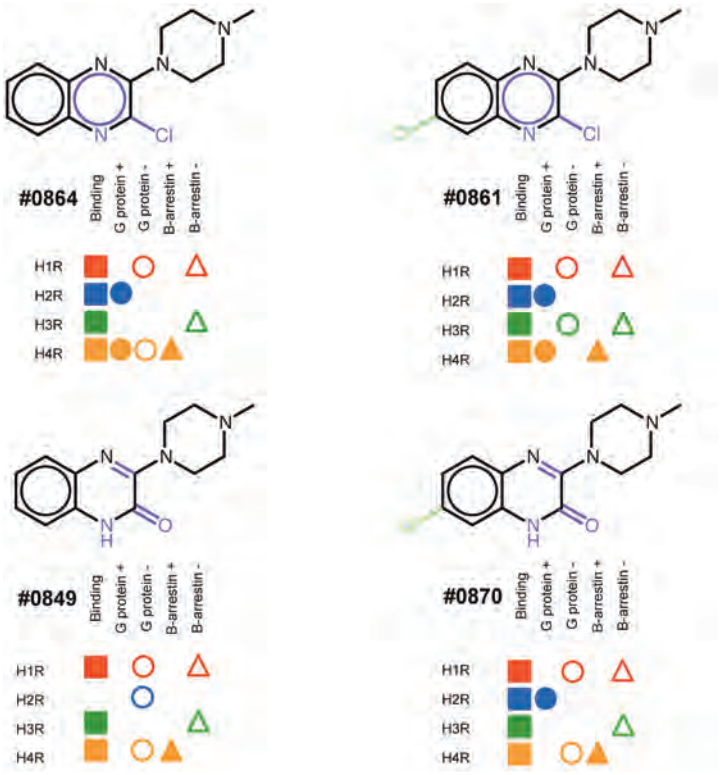
Unbiased and biased efficacy switches between receptor subtypes

In addition to fragments with comparable efficacy profiles on the histamine receptor subtypes, a large selection of fragments showed opposed effects at the different subtypes (i.e. agonists vs antagonists, biased vs unbiased). In this section we illustrate remarkable trends or extraordinary fragments.

Four structural (quinoxaline) analogues show minor structural differences, but have different efficacies at the histamine receptor subtypes. Fragments #0864[145] and #0861 are unbiased H_4R agonists, whereas fragments #0849 and #0870 are H_4R β-arrestin biased agonists (Figure 5-8). Chlorine atom substitution in #0861 results in unbiased H_3R antagonism, whereas chorine atom substitution in #0870 results in the gain of H_2R binding and H_2R G protein biased agonism. Interestingly, both #0864 and #0861 (with and without chlorine atom) possess H_2R G protein biased agonism. None of the structural changes influenced H_1R antagonism by the shown fragments (Figure 5-8). Interestingly, quinoxaline VUF10214 (Supplementary figure 5-I) was previously identified as β-arrestin biased H_4R ligand[175]. The structural difference between the unbiased and β-arrestin biased H_4R fragments is a substituent change from a chlorine atom (unbiased) into an oxygen atom (β-arrestin biased) as well as replacement of an hydrogen bond accepting ring nitrogen atom (unbiased) by a hydrogen bond donating NH group (β-arrestin biased) (Figure 5-8). These differences presumably alter the binding mode in such a way that the conformational changes are not large enough to initiate G protein activation, but subtle enough for β-arrestin recruitment. This is in line with our previous observations regarding the β-arrestin-biased H_4R indolcarboxamides, where also G protein activation required additional ligand-receptor interactions compared to β-arrestin recruitment[266]. Site-directed mutagenesis studies are now needed to identify ligand-receptor interactions and ultimately pinpoint specific amino acids or receptor regions responsible for biased receptor activation.

A large number of the biased H_3R G protein agonist target H_4R either as β-arrestin-biased agonists or as unbiased H_4R agonists. These G protein biased H_3R hits seem to be full biased fragments, as the majority of them antagonized RAMH-induced β-arrestin recruitment to the H_3R. Interestingly, these fragments (or their analogs) have been previously described as N-Substituted Piperidinyl Alkyl Imidazoles[295] or conformationally constrained agonists for the H_3R in CRE reporter gene assays[279]. Although the β-arrestin-biased and unbiased H_4R agonist structures are not novel for the H_4R, they are not all functionally characterized. We now show that these structures could be very interesting since they are either β-arrestin biased or unbiased at the H_4R, and G protein biased at the H_3R. Such subtle differences between structures and consequently large efficacy changes are extremely useful to elucidate the binding mode for biased compounds in the histamine receptor subtypes.

FIGURE 5-8. Structure activity relationships for multi-target fragments.



Four structural analogues show minor structural changes, but have diverse efficacies. Purple structure parts indicate structural changes between top and bottom fragments, whereas green indicates the chlorine atom substitution between left and right fragments. Efficacy profiles for each fragment are indicated below the structure.

CONCLUDING REMARKS

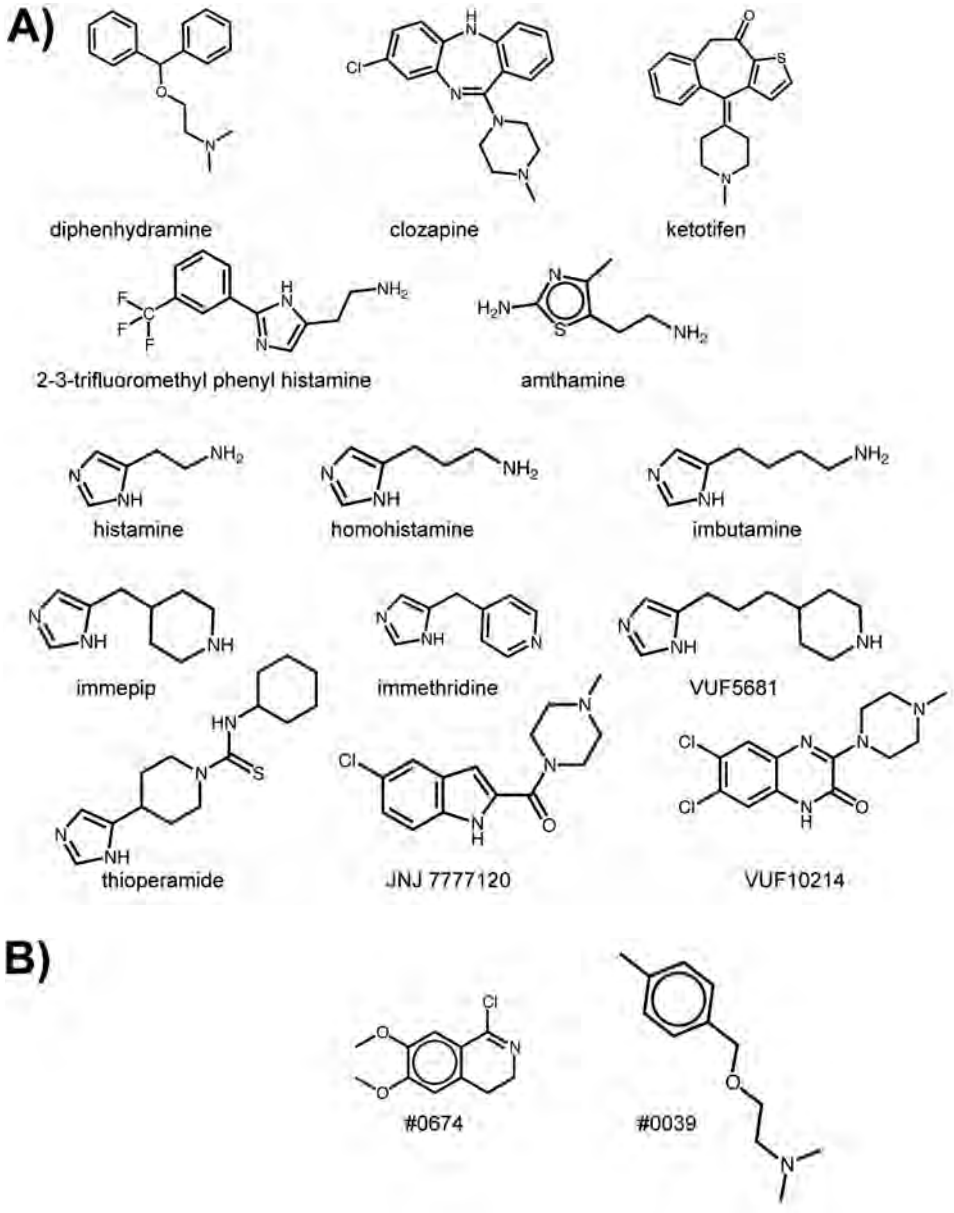
In this study we screened a fragment library in a radioligand binding assay and two distinct functional assays: G protein signalling and β-arrestin recruitment. All four histamine receptors showed agonist-induced β-arrestin recruitment. Although previous research suggested a role for β-arrestin in H_1R[296] and H_2R[297] desensitization and internalization, this is to our knowledge the first time that agonist-induced β-arrestin2 recruitment to H_1R, H_2R and H_3R is reported.
We identified receptor specific and overlapping fragments with agonist or antagonist activity at the four histamine receptors subtypes. Besides known histaminergic ligands a substantial amount of novel fragments could be identified. This does not only include yet unknown chemical structures, but also fragments with novel (biased) efficacies.

By comparing efficacy values in both assay, we were able to identify biased fragments for all histamine receptor subtypes. G protein biased ligands were found for H_2R, H_3R and H_4R and β-arrestin biased fragments were observed on H_1R and H_4R. Importantly, we could retrieve previously identified β-arrestin biased H_4R ligands. However, a substantial amount of novel biased fragments is also highlighted in the current studies. Further investigation into these new scaffolds in combination with the identified efficacy profiles will increase our knowledge of the structural mechanism behind biased histamine receptor signalling.

Some caution is however required, as it is dangerous to qualify a fragment as biased based on a single concentration screen. Fragments could well have different potency values depending on the assay type used. Consequently, screening at a single concentration that is hovering around the EC_{50} can easily results in fragments showing either full or no activity. Therefore our future efforts aim to further validate our findings with concentration response curves and/or inhibition data.

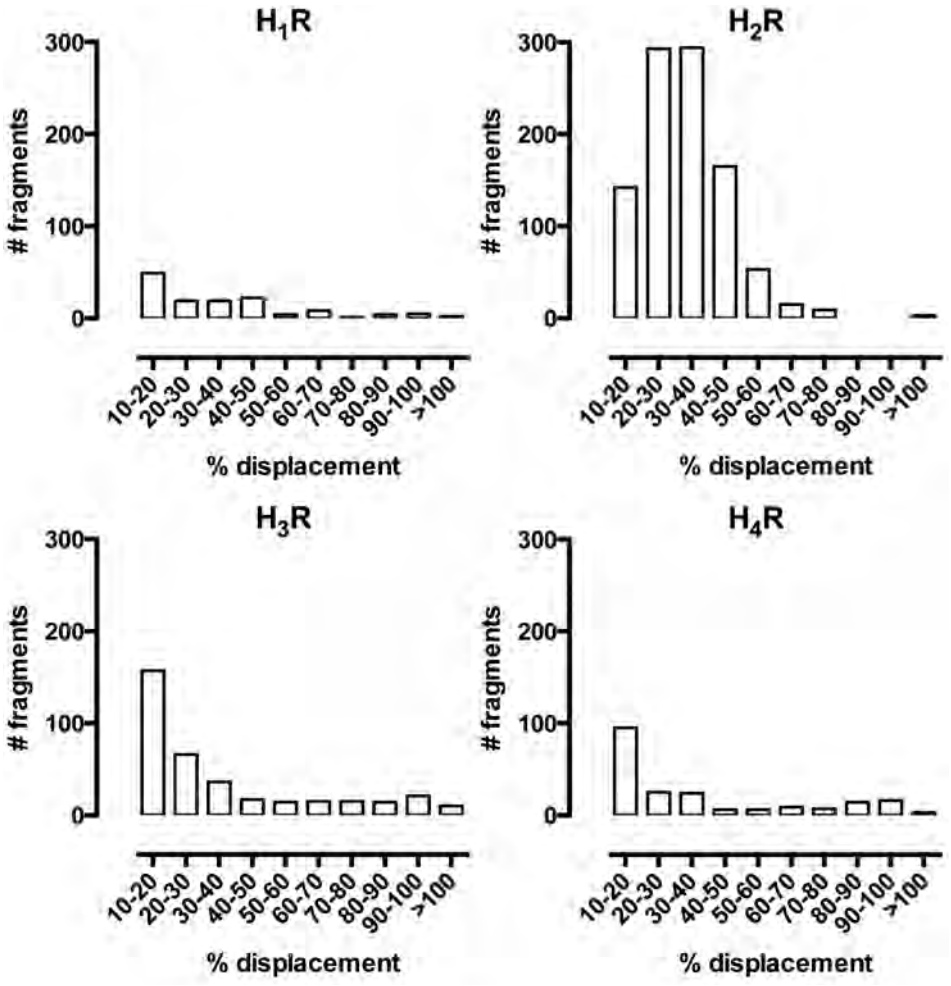
Finally, there is still an extensive amount of information that can be extracted from our dataset. New scaffolds and allosteric fragments could function as new starting points for future research.

SUPPLEMENTARY FIGURE 5-I. Chemical structures of described compounds.



A) Known and previously described H_1R-H_4R ligands. B) Fragments described in this manuscript that have not been published previously.

SUPPLEMENTARY FIGURE 5-II. Fragment distribution plots of radioligand binding assay.



Fragments that displace the radioligands from the histamine receptors show different displacement percentages. Amount of fragments is plotted against their normalized displacement.